

## Amendments to the Specification

Please replace the paragraphs at the indicated page and line numbers with the paragraphs set forth below.

(Page 23, lines 17-19)

### Brief Description of the Drawings

Some embodiments of the invention will now be described with reference to the accompanying drawings in which:

(Page 23, line 24 through page 24, line 12)

Figs. 2A-2D ~~gives~~ give the amino acid sequence comparison of the KS domains and the CLF domains of representative Type II PKS gene clusters. The active site Cysteine (C) of the KS domains is arrowed in the Figure and aligns with the Glutamine (Q) or glutamic acid (E) of the CLF domains. The abbreviations used, and the relevant Genbank/EMBL accession numbers are: GRA: granaticin from *Streptomyces violaceoruber* (X63449); HIR: unknown polyketide from *Saccharopolyspora hirsuta* (M98258); ACT, actinorhodin from *Streptomyces coelicolor* (X63449); CIN: unknown polyketide from *Streptomyces cinnamonensis* (Z11511); VNZ: jadomycin from *Streptomyces venezuelae* (L33245); NOG: anthracyclines from *Streptomyces nogalater* (Z48262); TCM: tetracenomycin from *S. glaucescens* (M80674); DAU: daunomycin from *Streptomyces* sp. C5 (L34880); PEU, doxorubicin from *Streptomyces peucetius* (L35560); WHI: WhiE spore pigment from *Streptomyces coelicolor* (X55942). From top to bottom, the sequences are SEQ ID NOs: 1-20, respectively.

(Page 24, lines 16-20)

Figs. 4A-4C ~~shows~~ show the amino acid sequence alignment of KSq-ATq loading didomains of the PKSs for niddamycin (SEQ

ID NO: 21), platenolide(spiramycin) (SEQ ID NO: 22), monensin (SEQ ID NO: 23), oleandomycin (SEQ ID NO: 24) and tylosin (SEQ ID NO: 25). The sequences for the monensin and oleandomycin loading didomains have not been previously disclosed.

(Page 24, line 26)

Fig. 7 shows the structures of two oligonucleotides. The forward and backward oligonucleotides are SEQ ID NOs: 26 and 27, respectively, and are shown as annealed with restriction enzyme sites.

(Page 25, lines 3-51)

All NMR spectra were measured in CDCl<sub>3</sub> using a Bruker 500MHz DMX spectrometer unless otherwise indicated and peak positions are expressed in parts per million (ppm) downfield from tetramethysilane. The atom number shown in the NMR structure is not representative of standard nomenclature, but correlates NMR data to that particular example.

#### HPLC methods

##### Method A

Column	Waters Symmetry 5_ C18 2.1mm X 150 mm
Flow	0.29 ml/min
Mobile phase	Gradient: A:B (22:78) to A:B (38:62) over 12 minutes, then to A:B (80:20) by minute 15. Maintain for 1 minute. Re-equilibrate before next sample. Where A = acetonitrile and B = 0.01M ammonium acetate in 10% acetonitrile and 0.02% TFA
Instrument	Acquired with Hewlett-Packard 1050 liquid chromatograph interfaced to

a VG Platform II mass spectrometer  
equipped with an APCI source

Method B

Waters Symmetry 5\_ C18 2.1mm X 150 mm  
0.29 ml/min

Gradient:28:72 acetonitrile:10mM NH4OAc

phase

to

in 18 minutes. 50:50 until 25 minutes. Back to 28:72,

ilibrate for 7 minutes

ment

Acquired with Hewlett Packard 1100 LC/MS

with

ource

Tap Water medium

glucose	5g/liter
tryptone	5g/liter
yeast extract	2.5g/liter
EDTA	36mg/liter
Tap water to 1L total volume	

ERY - P medium

dextrose	50g/liter
Nutrisoy <sup>TM</sup> flour	30g/liter
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	3g/liter
NaCl	5g/liter
CaCO <sub>3</sub>	6g/liter
Tap water to 1L total volume	
pH adjusted to 7.0	

(Page 26, lines 13-28)

The following synthetic oligonucleotides: 5'-

CCATATGGCCGCATCCGCGTCAGCGT-3' (SEQ ID NO: 28) and 5'-

GGCTAGCGGGTCCTCGTCCGTGCCGAGGTCA-3' (SEQ ID NO: 29)

are used to amplify the DNA encoding the putative monensin-producing loading module using a cosmid that contains the 5' end of the putative monensin-producing PKS genes from *S. cinnamomensis* or chromosomal DNA of *S. cinnamomensis* as template. The PCR product of 3.3 kbp is purified by gel electrophoresis, treated with T4 polynucleotide kinase and ligated to plasmid pUC18, which has been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid pPFL40. Plasmid pPFL40 was identified by restriction pattern and sequence analysis.

(Page 27, lines 1-16)

Plasmid pHD30His is a derivative of pNEWAVETE (PCT/GB97/01810) which contains the avermectin loading module, erythromycin extension modules 1 and 2 and the erythioesterase domain. Plasmid pNEWAVETE was cut with *Eco*RI and *Hin*DIII and a synthetic oligonucleotide linker was inserted that encodes the addition of a C-terminal polyhistidine tail to the polypeptide. The following oligonucleotides:

5'-AATTCACATCACCATCACCATCACTAGTAGGAGGTCTGGCCATCTAGA-3' (SEQ ID NO: 30) and

5'-AGCTTCTAGATGGCCAGACCTCCTACTAGTGATGGTGATGGTGATGTG-3' (SEQ ID NO: 31)

were annealed together and the duplex was ligated to *Eco*RI- and *Hin*DIII-cut pNEWAVETE. The resulting plasmid was cut with *Nde*I and *Xba*I and ligated into plasmid pCJR24 that had been previously cut with same two enzymes, to produce plasmid pND30His.

(Page 28, line 13 through page 30, line 37)

The culture *Saccharopolyspora erythraea* NRRL2338(pPFL43), constructed with the wild-type loading domain displaced by a monensin loader-D1TE DNA insert, produced as described in

Example 2, was inoculated into 30ml Tap Water medium with 50 ug/ml thiostrepton in a 300ml Erlenmeyer flask. After three days incubation at 29°C, this flask was used to inoculate 300 ml of ERY-P medium in a 300 ml flask. The broth was incubated at 29°C at 200 rpm for 6 days. After this time, the whole broth was adjusted to pH 8.5 with NaOH, then extracted with equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness at 45°C under a nitrogen stream using a Zymark TurboVap LV Evaporator, then reconstituted in 0.0625 volumes methanol to concentrate the extract 16-fold. The structures of the products were confirmed by LC/MS, Method A. A 4.0 min retention time peak was observed as the major component, with  $m/z$  value of 720 (M+H)<sup>+</sup>, required for 13-methyl-erythromycin A. A second peak was observed with a retention time of 6.4 min and with  $m/z$  value of 704 (M+H)<sup>+</sup>, required for 13-methyl-erythromycin B.

#### **Example 4**

#### **Production and Recovery of 13-methyl-erythromycin A and B using *Sacch. erythraea* NRRL-2338 (pPFL43) at 8L scale**

*Saccharopolyspora erythraea* NRRL2338 (pPFL43) was inoculated into 1000mls Tap Water medium with 50 µg/ml thiostrepton in a 2.8l Fernbach flask. After three days incubation at 29°C, this flask was used to inoculate 8l of ERY-P medium in a 14l Microferm fermentor jar (New Brunswick Scientific Co., Inc., Edison, NJ). The broth was incubated at 28°C with an aeration rate of 8l/min, stirring at 800 rpm and with pH maintained between 6.9 and 7.3 with NaOH or H<sub>2</sub>SO<sub>4</sub> (15%). Water was added to maintain volume at the 24 hour volume level. The fermentation was continued for 167 hours. After this time, presence of 13-methyl- erythromycin A and B were confirmed by adjusting a broth sample from the fermentor to pH 8.5 with NaOH, then extracting with equal volume of

ethyl acetate. The ethyl acetate extract was evaporated to dryness at 45°C under a nitrogen stream using a Zymark TurboVap LV Evaporator, then reconstituted in 0.25 volumes methanol to concentrate the extract 4-fold. The structures of the products were confirmed by LC/MS, Method A. A 4.1 min retention time peak was observed as the major component, with  $m/z$  value of 720 (M+H)<sup>+</sup>, required for 13-methyl-erythromycin A. A second peak was observed with a retention time of 6.6 min and with  $m/z$  value of 704 (M+H)<sup>+</sup>, required for 13-methyl-erythromycin B.

About 35 liters of broth containing approximately 2.8 grams of 13-methyl- erythromycin A were processed for recovery of product. Broth was filtered through a pilot sized Ceraflo ceramic unit and loaded onto a 500ml XAD-16 resin column. The product was eluted using 100% methanol. A 175ml CG-161 adsorption column was prepared and equilibrated with 20% methanol/water. A portion of the product solution was adjusted to 20% methanol and loaded onto the column, no breakthrough of product was observed. Washing of the column with up to 40% methanol/water failed at removing any significant level of impurities. Elution with 50% methanol/water achieved chromatographic separation of the product from the two major impurities, 13-methyl-erythromycin B and a degradation product, 13-methyl-dehydroerythromycin A. The purest cuts were combined and reduced in volume by approximately 75% using evaporation to achieve <10% methanol concentration. To enhance 13-methyl-erythromycin A extraction, solid sodium bicarbonate was added until a total concentration of 250mM was obtained. The aqueous product layer was extracted 2x with methylene chloride, using one-half the total volume each time. The volume was reduced to light yellow solids by evaporation. The 13-methyl-erythromycin A was purified by dissolving the crude crystals into methylene chloride at ambient temperature and diluting to 15% methylene chloride with hexane. The cloudy solution is placed at -10°C for ~30 minutes when the liquid is

decanted to a 2<sup>nd</sup> flask, leaving the majority of impurities behind as an oil. The flask is left overnight at -10°C, followed by filtration of off-white 13-methyl-erythromycin A crystals the next day. Approximately 300 milligrams of 13-methyl-erythromycin A were isolated from the partial work-up of the 351 broth volume.

Approximately 100 grams of evaporated mother liquor were utilized further to isolate 13-methyl-erythromycin B. Residual 13-methyl-erythromycin A was removed with repetitive extraction of the initial sample with aqueous acetic acid (pH 5). The subsequent methylene chloride layer was chromatographed on 700 g of silica gel using 20% methanol in methylene chloride. The 13-methyl-erythromycin B enriched fractions, as determined by LC/MS, were combined and evaporated to yield ~11.0 grams of dark oil. The oil was dissolved in a minimal amount of methanol and loaded onto 500 ml of Amberchrom CG-161 resin. The 13-methyl-erythromycin B was eluted at 2 bed volumes per hour with 40% methanol in deionized water. One bed volume fractions were collected and assayed by LC/MS. Fractions 42 through 62 were combined, diluted to ~20% methanol with deionized water, and neutralized to pH 7.5 with sodium bicarbonate. The resulting solution was extracted once with 41 of methylene chloride, concentrated to ~500 ml, and dried over anhydrous magnesium sulfate. After removal of the MgSO<sub>4</sub> by filtration the filtrate was evaporated to give ~110 mg of light brown solids. The 110 mg of crude 13-methyl-erythromycin B was dissolved in ~ 3.0 milliliters of HPLC grade acetonitrile and loaded onto a 20cm x 20cm, 2mm thick, silica gel preparative thin layer chromatography (PTLC) plate. The plate was developed with 60:40 methanol:acetonitrile. The desired portion of silica from the PTLC plate (iodine visualisation) was removed and extracted with HPLC grade acetone. The acetone extract was evaporated to give 12.1 mg of clear solid.

Identification of the 13-methyl-erythromycin A and 13-

methyl-erythromycin B samples were confirmed by mass spectroscopy (LC/MS Method B) and NMR spectroscopy. The 13-methyl-erythromycin A sample peak had a 4.7 min retention time, with  $m/z$  value of 720  $(M+H)^+$ , required for 13-methyl-erythromycin A. The 13-methyl-erythromycin B sample peak had a 7.6 min retention time, with  $m/z$  value of 704  $(M+H)^+$ , required for 13-methyl-erythromycin B.

(Page 34, lines 1-2)

#### **Example 5**

#### **Construction of plasmid pPFL35**

(Page 34, lines 12-18)

A 411 bp DNA segment of the *eryAI* gene from *S. erythraea* extending from nucleotide 1279 to nucleotide 1690

(Donadio, S. et al., Science (1991) 2523:675-679) was

amplified by PCR using the following synthetic

oligonucleotide primers:-

5'-TGGACCGCCGCCAATTGCCTAGGCGGGCCGAACCCGGCT-3' (SEQ ID NO: 32) and 5'-CCTGCAGGCCATCGCGACGACCGCGACCGGTTTCGCC-3' (SEQ ID NO: 33).

(Page 36, lines 6-23)

A DNA segment encoding the KSq domain from the *oleAI* gene of *S. antibioticus* extending from nucleotide 1671 to nucleotide 3385 was amplified by PCR using the following synthetic oligonucleotide primers:-

5'-CCACATATGCATGTCCCCGGCGAGGAA-3' (SEQ ID NO: 34) and 5'-CCCTGTCCGGAGAAGAGGAAGGCGAGGCCG-3' (SEQ ID NO: 35)

and chromosomal DNA from *Streptomyces antibioticus* as a



template. The PCR product was treated with T4 polynucleotide kinase and ligated to plasmid pUC18, which had been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL31. The new *Nde* I site bordering the insert is adjacent to the *Eco* RI site of the pUC18 polylinker while the new *Bsp* EI site borders the *Hin* dIII site of the linker region. Plasmid pPFL31 was identified by restriction and sequence analysis.

(Page 37, line 24 through page 38, line 18)

#### **Example 7**

#### **Production of 13-methyl-erythromycin A and B using *Sacch. erythraea* NRRL-2338 (pPFL35)**

The culture *Saccharopolyspora erythraea* NRRL2338(pPFL35), constructed with the wild-type loading domain displaced by an oleandomycin KSQ-rapamycin AT2-D1TE DNA insert, prepared as described in Example 6, was inoculated into 30ml Tap Water medium with 50 ug/ml thiostrepton in a 300ml Erlenmeyer flask. After two days incubation at 29<sup>0</sup>C, this flask was used to inoculate 300 ml of ERY-P medium in a 300 ml flask. The broth was incubated at 29<sup>0</sup>C at 200 rpm for 6 days. After this time, the whole broth was adjusted to pH 8.5 with NaOH, then extracted with an equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness at 45<sup>0</sup>C under a nitrogen stream



**erythraea NRRL-2338 (pPFL44)**

The culture *Saccharopolyspora erythraea* NRRL2338 (pPFL44), constructed with the wild-type loading domain displaced by spiramycin loader-D1TE DNA insert, was inoculated into 30ml Tap Water medium with 50 ug/ml thiostrepton in a 300ml Erlenmeyer flask. After three days incubation at 29<sup>0</sup>C, this flask was used to inoculate 300 ml of ERY-P medium in a 300 ml flask. The broth was incubated at 29<sup>0</sup>C at 200 rpm for 6 days. After this time, the whole broth was adjusted to pH 8.5 with NaOH, then extracted with equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness at 45<sup>0</sup>C under a nitrogen stream using a Zymark TurboVap LV Evaporator, then reconstituted in 0.0625 volumes methanol to concentrate the extract 16-fold. The structures of the products were confirmed by LC/MS, Method A. A 4.0 min retention time peak was observed as the major component, with *m/z* value of 720 (M+H)<sup>+</sup>, required for 13-methyl-erythromycin A (C<sub>36</sub>H<sub>65</sub>NO<sub>13</sub>). A second peak was observed with a retention time of 6.4 min and with *m/z* value of 704 (M+H)<sup>+</sup>, required for 13-methyl-erythromycin B (C<sub>36</sub>H<sub>65</sub>NO<sub>12</sub>).

(Page 41, lines 9-22)

The approximately 1.47 kbp DNA fragment of the *eryAI* gene of *S. erythraea* was amplified by PCR using as primers the synthetic oligonucleotides:

5'-TACCTAGGCCGGGCCGGACTGGTCGACCTGCCGGGTT-3' (SEQ ID NO: 38)  
and 5'-ATGTTAACCGGTCGCGCAGGCTCTCCGTCT-3' (SEQ ID NO: 39) and plasmid pNTEP2 (Oliynyk, M. et al., Chemistry and Biology (1996) 3:833-839; WO98/01546) as template. The PCR product

was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK02 was identified by its restriction pattern and DNA sequencing.  
(Page 41, line 26 through page 42, line 9)

The approximately 1.12 kbp DNA fragment of the *eryAI* gene of *S. erythraea* was amplified by PCR using as primers the synthetic oligonucleotides:

5'-ATGTTAACGGGTCTGCCGCGTGCCGAGCGGAC-3' (SEQ ID NO: 40) and  
5'-CTTCTAGACTATGAATTCCTCCGCCAGC-3' (SEQ ID NO: 41) and  
plasmid pNTEPH as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK03 was identified by its restriction pattern and DNA sequencing.

(Page 45, line 23 through page 46, line 10)

The approximately 2.2 kbp DNA segment of the *rapB* gene of *S. hygroscopicus* encoding the reductive loop of module 10 was amplified by PCR using as primers the synthetic oligonucleotides:

5'-TAAGATCTTCCGACGTACGCGTTCCAGC-3' (SEQ ID NO: 42) and  
5'-ATGCTAGCCACTGCGCCGACGAATCACCGGTGG-3' (SEQ ID NO: 43) and  
as template an approximately 7 kbp fragment, which has been obtained by digestion of cosmid cos 26 (Schwecke, T. et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:7839-7843) with ScaI and SphI. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with

alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK121.1 was identified by its restriction pattern and DNA sequencing.

(Page 46, line 26 through page 47, line 13)

The approximately 6.1 kbp DNA segment of the erythromycin PKS gene cluster of *S. erythraea* encoding the DNA fragment from the beginning of the ACP of module 2 to the beginning of the ACP of module 3 was amplified by PCR using as primers the synthetic oligonucleotides:

5'-TACCTGAGGGACCGGCTAGCGGGTCTGCCGCGTG-3' (SEQ ID NO: 44) and  
5'-ATGCTAGCCGTTGTGCCGGCTCGCCGGTCTGGTCC-3' (SEQ ID NO: 45) and  
plasmid pBAM25 (published pBK25 by Best, D J et al. *Eur J Biochem* (1992) 204: 39-49) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK50 was identified by its restriction pattern and DNA sequencing.

(Page 52, lines 2-19)

The approximately 1.8 kbp DNA segment of the monensin PKS gene cluster of *Streptomyces cinnamonensis* encoding part of the ACP of the loading module and KS of module 1 was amplified by PCR using as primers the synthetic oligonucleotides:

5'-CGTTCCTGAGGTCGCTGGCCCAGGCGTA-3' (SEQ ID NO: 46)

5'-CGAAGCTTGACACCGCGGCGCGCGCGG-5' (SEQ ID NO: 47)

and a cosmid containing the 5' end of the monensin PKS genes from *S. cinnamonensis* or alternatively chromosomal DNA of *S. cinnamonensis* as template. The PCR product was treated with

T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pPFL45 was identified by its restriction pattern.

(Page 53, lines 9-29)

For the PCR amplification for plasmid pM009, the following synthetic oligonucleotides were used as mutagenic primers, one containing a MunI site and the other a PstI site:

5' -GCGCGCCAATTGCGTGACATCTCGAT- 3' (SEQ ID NO: 48)

and 5' -CCTGCAGGCCATCGCGACGACCGCGACCGGTTGCGCCG- 3' (SEQ ID NO: 49)

For the PCR amplification for plasmid pM010, the following synthetic oligonucleotides were used as mutagenic primers, one containing a HindIII site and the other an EcoRV site:

5' -GTCTCAAGCTTCGGCATCAGCGGCACCAA- 3' (SEQ ID NO: 50)

and 5' -CGTGCGATATCCCTGCTCGGCGAGCGCA-3' (SEQ ID NO; 51)

For the PCR amplification for plasmid pM013, the following synthetic oligonucleotides were used as mutagenic primers, one containing a PstI site and the other a HindIII site:

5' -GATGGCCTGCAGGCTGCCCCGGCGGTGTGAGCA- 3' (SEQ ID NO: 52)

and 5' -GCCGAAGCTTGAGACCCCCGCGCGGTCGC- 3' (SEQ ID NO: 53)